STUDIES ON TOXOPLASMOSIS II.

SOME OBSERVATIONS ON STRAIN "HT" WHICH WAS ISOLATED FROM A HARE (*LEPUS TIMIDUS AINU*) IN SAPPORO

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INTRODUCTION

As already reported, the authors could isolate a toxoplasma strain from the lung materials of one hare which died of toxoplasmosis. Recently in Japan the isolations of toxoplasma have been successively described by several workers from various species of animals such as cat, dog, swine, rat, guinea pig as well as from the human being. The present strain which is designated HT, is the first hare strain to be found in this country. Since isolation it is being maintained by mouse passage with intraperitoneal injections into General Purpose Mice of the National Institute of Health, Bethesda, Md., U. S. A.

Nowadays, it is not sufficient to regard it as *Toxoplasma gondii* on morphological grounds alone. Accordingly the present authors made some observations on its virulence for some laboratory animals and on the serological behaviour of this strain HT, in comparison with the RH strain which was supplied by Dr. TSUNEMATSU, Institute for Infectious Diseases, Tokyo University in the form of the 1196 mouse passage.

In the course of this examination, the authors' strain HT exhibited some interesting characteristics, namely non-antigenicity in the dye test for long time after its isolation, in spite of its stimulating the high titre of the dye test antibody only reacting with RH antigen by rabbit inoculations.

The above stated serological feature was always observed till about the 60th generation of mouse passage. However this feature of the present strain recently changed, although the reasons are not understood and revealed the same sort of positive reactions in vitro as are shown by RH strain.

This fact observed by the present authors seems to be very important and must be kept in mind when serological identification of toxoplasma is being made.

The data obtained are briefly described in the present paper.

MATERIALS AND METHODS

1. Dye Test

The technics employed in the present experiments were those described by SABIN and FELDMAN as slightly modified by HASEGAWA et al. The equal volume of antigen which was suspended in the accessory factor serum containing heparin to the amount of 0.2% was added to the small test tubes containing 0.1 ml of each serially 2-fold diluted test serum and was kept at 37°C for 1 hour. After refrigeration, 0.05 ml of dye was added and the stained parasites were examined by routine method.

Preparation of dye: 10 ml of alkaline soda-borate buffer of pH 11 (9.73 ml of 0.53% Na_2CO_3 + 0.27 ml of 1.917% $Na_2B_4O_7 \cdot 10H_2O$) with the addition of 3 ml of saturated alcoholic solution of methylene blue medicinale.

The authors always utilized 3rd day mouse peritoneal exudate of strain RH, however in case of strain HT, they sometimes could not but employ the 4th day, sometimes the 5th day, because of the poor multiplication of the parasites in animal body. In this case even by using 5th day peritoneal exudate, in the course of the test the authors had never encountered any difficulties such as were often experienced by many workers probably due to neutralization by the presence of antibody in mouse peritoneal fluid.

2. Complement-fixation Test

1) Antigen

The antigens employed in the present report were prepared following SABIN from the chorioallantoic membranes of embryonated eggs infected respectively with the strain RH and with the strain HT. Centrifuged clear supernatant from the tissue culture of L-cells infected with these toxoplasmic strains was also employed. The control antigens were prepared from non-infected materials by the same procedures.

2) Sera

The sera employed were those from the rabbit which were infected by abdominal or intracerebral inoculation of mouse peritoneal exudate of strain HT for the purpose of examining its virulence for the laboratory animals. However as for the sera of strain RH, because of its high virulence, preliminary injections with the dead parasites were carried out before subcutaneous or abdominal inoculations of live organisms.

The animals were sacrificed when sera reached to the sufficiently high titre for serodiagnosis. The test sera were diluted 1 : 2 with saline and heated at 60°C for 20 minutes before examination.

3) Technics

The test on the whole followed the procedure of the American Military Medical School. Each 0.25 ml of serial 2-fold dilution of serum and preliminary titrated antigen were placed in small test tubes with 0.5 ml of the predetermined dilution of guinea pig serum containing 2 full units of complement. The tubes were left in a refrigerator (at about 5°C) for about 16 hours. After this, 0.5 portions of the sensitized sheep erythrocytes representing

a mixture of equal parts of a 3% suspension of washed red cells and hemolysin diluted to contain 3 units per 0.25 ml were added and then the entire material was incubated in a water bath at 37°C for 30 minutes.

The results were read immediately.

Results

1. Pathogenicity of the Strain HT for Mice

The strain HT caused death in mice first after the 4th generation of blind passages of original materials. The days elapsed before death of 3 mice were respectively 10, 14 and 14. After this by peritoneal or intracerebral inoculation of the infected peritoneal fluid, death always occurred within 5~9 days; the authors could not recognize the shortening of survival days of mice by increasing of animals passages which were often observed by workers such as TSUNEMATSU in DR strain recovered from Norway rat. At the 24th and 28th passages, virulence for mice was examined. The data obtained are shown in table 1.

TABLE 1. *Pathogenicity of the Strain HT for Mice*
(General Purpose, N. I. H, Bethesda)

NO. OF MOUSE PASSAGES	NUMBER OF PARASITES INOCULATED (I. P.)				
	27×10^4	27×10^3	27×10^2	27×10^1	27
24	◎$_7$ ◎$_8$	◎$_8$ ◎$_8$	◎$_9$ ◎$_9$	◎$_9$ ◎$_{11}$	○ ○
			3×10^3	3×10^2	3×10^1
28			◎$_{12}$ ◎$_{14}$	◎$_9$ ◎$_{12}$	◎$_{12}$ ○

◎$_7$ means death at 7th day after inoculation.

The results indicated that the present strain is highly pathogenic for mice as well as the strain RH, that is to say, death usually occurs with several hundreds or tens of number of parasites. However, as is indicated in table 2, the number of parasites per ml of the peritoneal washings with two ml of saline at the death were surprisingly few as compared with the findings in the strain RH, though there seems to be not very much difference of survival days between them. This feature continued for several tens of passages; however, after about the 60th generation or less of mouse the number of parasites in the peritoneal washing often increased over several ten millions and after the 70th or the 80th generation mouse passage there seem to be no significant differences between the newly isolated HT and the standard RH strain.

2. Virulence for the Other Laboratory Animals

It is generally said that the strain highly pathogenic for mice also shows the greatest virulence for other laboratory animals such as guinea pig and rabbit etc., and that enhancement in virulence by mouse passage does occur not only in mice but also in other hosts.

TABLE 2. *Survival Days and Number of Parasites in the Peritoneal Washings of Mice Inoculated with Two Strains*

STRAIN	NUMBER OF PARASITES INOCULATED (I. P.)								
	200,000				500,000				
	Survival Days	* Number of Parasites in Peritoneal Washings			Survival Days	* Number of Parasites in Peritoneal Washings			
HT (39th~46th mice generation)	5, 5, 6, 6, 6,	760	357	162	5, 5, 6, 6, 6,	420	164	120	
	6, 6, 5, 5, 5,	796	564	105	6, 6, 6, 6, 6,	320	156	94	
	5, 6, 6, 6, 6,	702	375	190	6, 6, 6, 6,	408	110		
	6, 6, 6,	624	241	345		410	208		
		229	229	239		350	592		
	(5.7)	226	452	145	(5.8)	228	187		
		134	187						
			(348)				(269)		
RH (1198th~1202nd mice generation)	5, 5, 5, 5, 5,	3,400	7,200	1,168	4, 5, 5, 5, 5,	3,520	6,100		
	5, 6, 6, 6, 6,	4,650	925	3,450	5, 5, 5, 5, 5,	7,500	5,500		
	7, 5, 5, 5, 5,	2,900	8,208		5, 5, 6, 6, 6,	4,100	3,500		
	5, 6, 6, 6, 6,	1,392	1,750		6,	2,400	2,968		
		2,368	945			1,510	8,000		
	(5.5)	4,320	2,000		(5.1)	4,010	1,168		
		1,840	2,500			1,400	2,000		
		5.200	1,350			4,200			
		2,576	1,168			2,430			
			(2,966)				(3,768)		

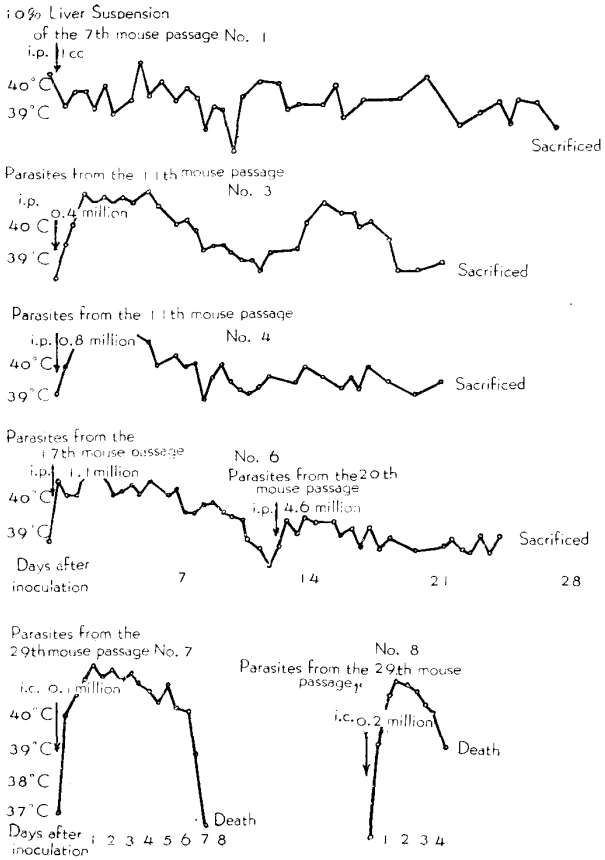
* indicate the parasite number (unit: ten-thousand) per ml of the peritoneal washings with 2 ml of physiological saline at death.

As already mentioned, the present strain showed high virulence for mice, however for guinea pig and rabbit the strain indicated considerably low virulence. As is indicated in fig. 1, the guinea pigs which were inoculated intraperitoneally with the present strain survived, although they revealed febrile reactions. The authors could not identify toxoplasma in the stained smear of each of the organ materials after 3~4 weeks observation period, except in the mesenteric lymph node of No. 3 which manifested mixed infection with spirochaeta-like organisms. However, in two guinea pigs which received 100 and 200 thousand of parasites by the intracerebral route, the death occurred in 4 and 8 days respectively.

For the rabbit, the present strain also exhibited low virulence. All rabbits inoculated intraperitoneally as well as intracerebrally, revealed only febrile reactions as indicated in fig. 2, although all the rabbits inoculated with strain RH, even by subcutaneous route, showed severe infection and died.

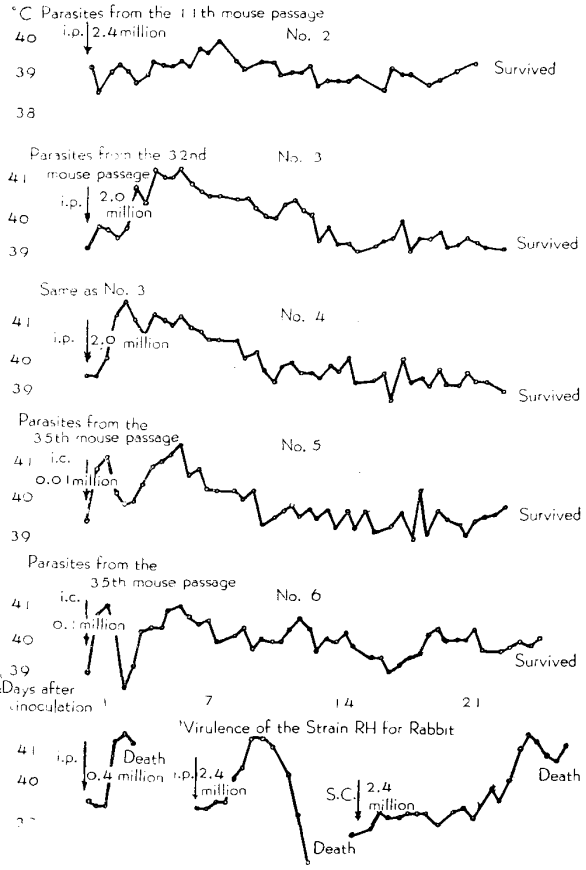
The rabbits Nos. 2, 3, 4, 5 and 6 were sacrificed respectively on the 65th, 144th, 46th,

FIG. 1. *Virulence of the Strain HT for Guinea Pig*



Remark: Except No. 1, the materials for injection were the parasites from the peritoneal washings of each mouse passage.

FIG. 2. *Virulence of the Strain HT for Rabbit*



Remark: The materials for injection were the parasites from the peritoneal washings of each mouse passage. The strain RH was tested between the 1204~1213 mouse passage.

57th and 144th day after inoculation. The postmortem examinations, however, showed no significant changes and the parasites were not identified in the stained smear of each organ material. Histopathologically, some of them showed very slight changes of meningitis (No. 5) and slight accumulation of glia cells scattered in very small numbers (No. 4), also the parasites were not detected. In these cases, the pathogenicity of toxoplasma was examined during the mouse passages from the 7th to 30th. As was already reported, for embryonated eggs, the strain now under study caused death by allantoic or yolk sac routes and showed characteristic lesions in the chorioallantoic membrane as in the strain RH.

3. Serological Observations

1. Dye test according to the method of SABIN and FELDMAN

The guinea pigs and rabbits which were inoculated with the present strain always

TABLE 3. *Result of Cross Dye-Test between the Isolated Strain HT and Control Strain RH*

1)

SERUM		HT NO. 1					HT NO. 2					RH NO. 1				RH NO. 2				ANTIGEN
Serum Dilution 2^N	N=	8	9	10	11	12	6	7	8	9	10	9	10	11	12	9	10	11	12	CONTROL
Antigen RH (1218)*		###	###	###	###	—	###	###	###	##	—	###	###	###	±	###	+	—	—	—
		93	96	89	82	36	96	95	83	71	25	97	99	92	50	90	53	43	23	11
Antigen HT (20)*		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		27	18	30	21	15	41	40	27	17	0	34	19	17	17	27	25	16	13	8

2)

SERUM		HT NO. 3									HT NO. 4								ANTIGEN
Serum Dilution 2^N	N=	5	6	7	8	9	10	11	12	13	5	6	7	8	9	10	11	12	CONTROL
Antigen RH (1206)*						###	###	###	+	—					###	###	###	##	—
						93	97	94	53	24					99	98	91	79	7
Antigen HT (46)*		—	—	—	—	—	—				—	—	—	—	—	—	—		—
		5	26	14	11	36	31				8	6	12	10	35	32	28		2

3)

SERUM		HT NO. 1						HT NO. 2					ANTIGEN CONTROL	HT NO. 3				ANTIGEN CONTROL	SWINE NO. 12							ANTIGEN
Serum Dilution 2^N	N=	8	9	10	11	12	13	6	7	8	9	10	CONTROL	8	9	10	11	CONTROL	1	2	3	4	5	6	7	CONTROL
														**					***							
Antigen HT (79)*		###	###	##	++	—	—	###	###	###	##	—	—	##	++	+	—	—	###	###	##	+				—
		99	99	88	72	35	29	94	99	99	89	35	6	84	70	60	48	0	94	91	85	10				7
Antigen RH (1216)*																			###	###	###	##	###	+	—	—
																			100	100	94	100	90	51	34	9

Remarks:
1. HT No. 1~No. 4 sera were obtained from the rabbits inoculated with the parasites from peritoneal fluid of each 7th, 11th, 32nd and 35th mouse passage.
2. Numerals under + or − indicate the number of unstained parasites in total 100.
3. * indicate the number of mouse passage after isolation.
4. ** indicate that HT antigen of the 57th mouse passages was employed, and *** that of the 65th.

developed cytoplasm-modifying antibodies reacting only with the strain RH antigen in high titre. In these rabbits the titres were generally found to be in 1 : 1,024～1 : 4,096 or more during the period of 2～3 weeks after inoculation.

As far as the present authors examined, the antibodies produced in rabbits with the present strain, in each 7th, 11th, 32nd and 35th mouse passages, react only to the strain RH. The experiments with the antigen HT always indicated negative results as listed in 1) and 2) of table 3. It is certain that this characteristic of the non-reactivity of this strain was maintained to at least the 46th mouse passage after its isolation. However, as indicated in 3) of table 3, this strain began to react with the same sera which formerly did not react at all in vitro. The fact was first observed when the test was made at the 57th mouse passage. After this, the strain always indicated antigenicity in vitro.

This observation seems to be very important in the identification of a toxoplasmic strain by use of dye test.

2. Complement-fixation test

In complement-fixation test both antigens of the strain indicated the same reacting range to the infected rabbit serum with the strain HT as is shown in table 4. These antigens were prepared from the chorioallantoic membranes following SABIN.

From the above stated facts, the HT strain is identified as *Toxoplasma gondii*.

TABLE 4. *Complement-fixation test*

Serum Dilution (HT. No. 4) 2^N N=	HT (56)*						RH (1215)*						CONTROL ANTIGEN		CONTROL SERUM	
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	6	7
6	4	4	4	4	4	3	4	4	4	4	4	1^+	0	0	0	0
7	4	4	4	4	4	1^-	4	4	4	4	2	0	0	0	0	0
8	4	4	4	4	2	0	4	4	4	4	0					
9	4	4	4	4	1^-	0	4	4	4	4	0					
10	4	4	4	1^-	0		4	4	1^-	1^-						
11	0	0	0	0			0	±	0	0						

* indicate the number of mouse passage with which the antigens were prepared following SABIN.

DISCUSSION

It is usually accepted that toxoplasmic strains are immunologically identical with each other. However by the dye test following SABIN and FELDMAN, there seem to be some differences in dye titre between two strains against one test serum, which are recognized by JACOBS and COOK or OHTSURU et al., and KOIKE et al. As the cause of this, KOIKE et al. suggested the presence of a strain specific antigen in minor degree, however JACOBS and COOK stated that this may be due to the different components of mouse peritoneal fluid which are influenced by the pathogenicity of each strain. However, from the above statements, the authors

had not learned that in the dye test there are some strains which do not reveal any antigenicity in vitro. Serological identifications are generally performed before not so many mouse passages after their adaptation for mice.

It is a very interesting and important problem that there are some strains such as HT which had not revealed any antigenicity in vitro until about the 60th mouse passage. Accordingly, the facts stated above must be remembered in connection with serological identification of the newly isolated strains.

The authors are unaware of the cause of this phenomenon, however it is certain that there had not occurred any antigenic variations, because the isolated strain of even the 7th mouse passage stimulated the antibody common with RH antigen in rabbit inoculation. Also any variation of virulence could not be noticed. Accordingly it is supposed that some substance which obstruct the reaction of antigen and antibody had been eliminated from the parasites or peritoneal fluid by some unknown factors.

SUMMARY

The hare strain HT of *Toxoplasma gondii* which was reported in the previous paper was tested immunologically and also pathogenicity for the laboratory animals and was examined in comparison with known toxoplasma strain RH. The data obtained are summarized as follows.

1. The strain HT indicated high virulence for mice and death occurred generally in 5～6 days after intraperitoneal inoculation, almost the same as the RH; however the number of parasites in mouse peritoneal fluid was very small in comparison with the strain RH.

2. For rabbit and guinea pig, the strain showed very low virulence. In rabbit, even resulting from intracerebral inoculation, only fever reaction occurred and the general conditions ran always normal afterward.

3. Immunologically, by the use of cross complement-fixation test, this strain was identified as *Toxoplasma gondii*.

4. At first, the isolated strain HT had revealed no reactivity in vitro in case of dye test with the rabbit sera infected with the strains HT and RH, however the isolated strain had showed the ability to produce cytoplasm-modifying antibody reacting with the antigen RH. After about more than 50 mouse passages, the strain revealed reactivity in vitro the same as the strain RH.

The above facts must be remembered for serological identification of the newly isolated strains by the dye test.

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